PPARδ Activation Promotes Stratum Corneum Formation and Epidermal Permeability Barrier Development during Late Gestation

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The goal of epidermal ontogenesis is to form a stratum corneum (SC), which is required for post-natal permeability barrier function. The regulation of epidermal ontogenesis is poorly understood, but nuclear hormone receptors have been shown to have an important function. As peroxisome proliferator-activated receptor-δ (PPARδ) is very abundant in fetal epidermis and PPARδ activation stimulates differentiation and permeability barrier formation in adults, we hypothesized that PPARδ might regulate epidermal ontogenesis. Treatment of fetal rat explants with the PPARδ ligand, GW 610742X, accelerates permeability barrier development, evidenced by a decrease in transepidermal water loss and an enhanced outside-in barrier function, attributable to the presence of more mature lamellar membranes in the SC and enhanced expression of loricrin and involucrin. Similarly, the intra-amniotic administration of GW 610742X also accelerates the formation of the SC and permeability barrier development. Finally, in PPARδ-deficient mice the formation of the SC and the expression of differentiation-related proteins were delayed on days 16.5 and 17.5 of gestation. However, at later stages (day 18.5 and after birth), there were no differences between wild-type- and PPARδ-deficient mice, indicating only a transient delay in epidermal ontogenesis. These studies show that PPARδ has a role in SC formation and permeability barrier development.

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INTRODUCTION

It is essential that before birth the skin forms a competent barrier, separating the outside hostile environment from the organism. One of the key barrier properties of the skin is the obstruction of the movement of water and electrolytes, that is, the permeability barrier. Without a competent permeability barrier, increased water loss occurs, leading to disturbances in fluid and electrolyte balance and hypothermia due to evaporative heat loss. Full-term infants have a normal permeability barrier with transepidermal water loss (TEWL) similar to that observed in adults (Hammarlund and Sedin, 1979; Harpin and Rutter, 1983). However, premature infants (<33 weeks gestation) show both increased TEWL and enhanced absorption of xenobiotics, indicative of abnormal permeability barrier function (Hammarlund and Sedin, 1979; Harpin and Rutter, 1983). This permeability barrier abnormality is a major source of morbidity and even mortality in very premature infants (<28 weeks gestation) (Cartlidge and Stewart, 1995).

The permeability barrier is localized to the outer layer of the epidermis, the stratum corneum (SC) (Elias and Menon, 1991; Feingold, 2007). The SC consists of corneocytes, keratinocytes (Kers) that have undergone terminal differentiation, surrounded by a neutral lipid-enriched extracellular matrix. The hydrophobic, extracellular lipid matrix provides the principal barrier to the transcutaneous movement of water and electrolytes. These lipid-enriched lamellar membranes in the SC contain predominantly cholesterol (CHOL), free fatty acids, and ceramides, which are derived from the exocytosis of lamellar body (LB) contents from the outermost stratum granulosum (SG) cells. However, the corneocytes provide a scaffold for these extracellular lipids, and therefore, abnormalities in corneocyte structure can also alter permeability barrier function. Keratinocytes undergo a complex pathway of differentiation, which culminates in Ker cornifi-

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Abbreviations: CHOL, cholesterol; EM, electron microscopy; FIL, filaggrin; H&E, hematoxylin and eosin; INV, involucrin; Ker, keratinocyte; KO, knock out; LB, lamellar body; LOR, loricrin; SC, stratum corneum; SG, stratum granulosum

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cation and in the formation of extracellular lipid-enriched lamellar membranes in the SC (Fuchs, 1990; Eckert *et al.*, 1997).

The SC and the permeability barrier develop late during the third trimester in both humans (Foster *et al.*, 1988) and in rodents. In the fetal rat a competent barrier is not present at day 19 of their 22 day gestation period (Aszterbaum *et al.*, 1992). However, by day 21, just before birth, a competent permeability barrier is formed. The emergence of a functional permeability barrier correlates with both the appearance of a multilayered SC and the formation of mature lamellar membranes in the SC interstices (Aszterbaum *et al.*, 1992). Moreover, structural proteins required for corneocyte formation, such as loricrin (LOR), filaggrin (FIL), and involucrin (INV), increase late in gestation in parallel with permeability barrier formation. The factors that regulate fetal SC development and permeability barrier formation are not well understood. Previous studies have shown that glucocorticoids, estrogen, and thyroid hormone stimulate, while testosterone delays SC formation and permeability barrier development (Aszterbaum *et al.*, 1992, 1993; Hanley *et al.*, 1996a, 1996b, 1997a, 1997b, 1998a).

Activators of peroxisome proliferator-activated receptor (PPAR)-α, -δ, and -γ have numerous effects on epidermal structure and function (Kuenzli and Saurat, 2003; Schmuth *et al.*, 2008). The addition of PPAR (α, δ, and γ) activators to cultured human Kers, and the topical application of these activators to adult mouse skin stimulate the expression of INV, LOR, and profilaggrin (Hanley *et al.*, 1998b; Komuves *et al.*, 2000; Westergaard *et al.*, 2001; Schmuth *et al.*, 2004), which are required for corneocyte formation. In addition, topical treatment of adult murine skin with PPAR (α, δ, and γ) activators improves permeability barrier homeostasis, due at least in part to increased LB density and secretion (Man *et al.*, 2006). In previous studies we have shown that activators of PPARα, but not PPARγ, stimulate fetal SC development and permeability barrier formation in both a fetal skin explant model that mimics intrauterine skin development, and following intra-amniotic administration (Hanley *et al.*, 1997b, 1999). Moreover, PPARα-deficient mice show a transient delay in the formation of the SC and a modest decrease in the expression of both INV and LOR, markers of Ker differentiation (Schmuth *et al.*, 2002). However, at birth, the SC and permeability barrier are normal in PPARα-deficient mice (Schmuth *et al.*, 2002).

Peroxisome proliferator-activated receptor-δ is the most abundant isoform of PPAR expressed in fetal epidermis (Braissant and Wahli, 1998; Michalik *et al.*, 2001). We therefore hypothesized that activation of PPARδ would have an important function in regulating fetal SC formation and permeability barrier development. In this article, we show that PPARδ activation stimulates SC formation and permeability barrier development in both the fetal skin explant model and following intra-amniotic administration. In addition, we show a delay in fetal SC formation in mice deficient in PPARδ, indicating that PPARδ has a function in barrier ontogenesis.

RESULTS

Activation of PPARδ accelerates SC formation in the fetal skin explant model

Our initial experiments determined the effect of the PPARδ activator, GW 610742X, on permeability barrier function. Skin explants from day 17 fetal rats were incubated in the presence of either GW 610742X or vehicle, and TEWL was measured gravimetrically after 2 days, as previously described (Hanley *et al.*, 1996c). As shown in Figure 1, GW 610742X treatment significantly reduced TEWL at 2 and 5 hours, compared with vehicle control ($P<0.05$, $n=5$–6), indicating that PPARδ activation stimulates permeability barrier formation in fetal rat explants.

To investigate the basis for these functional changes, we next assessed the histological alterations in the epidermis of fetal rat explants following treatment with the PPARδ activator. During the third trimester (days 17–21), rat epidermis displays a dramatic change in morphology (Aszterbaum *et al.*, 1992) with a marked increase in the number of epidermal cell layers of the epidermis and the appearance of a SC. In parallel with these morphological changes, several Ker differentiation markers, including INV, LOR, and FIL, are expressed in the rat epidermis on days 17, 19, and 20, respectively (Bickenbach *et al.*, 1995; Hardman *et al.*, 1998). Consistent with our previous report (Aszterbaum *et al.*, 1992), after 2 days of incubation, skin explants from 17 day fetal rats (days 17 + 2) lacked a multilayered SG and a distinct SC, a pattern corresponding to day 19 of gestation (Supplementary Figure S1, a). In contrast, both a multilayered SG and layers of distinct SC were present in epidermis incubated with GW 610742X (Supplementary Figure S1, b-c). These results indicate that the acceleration in permeability barrier formation induced by PPARδ activation is associated with the appearance of more mature epidermal morphology.

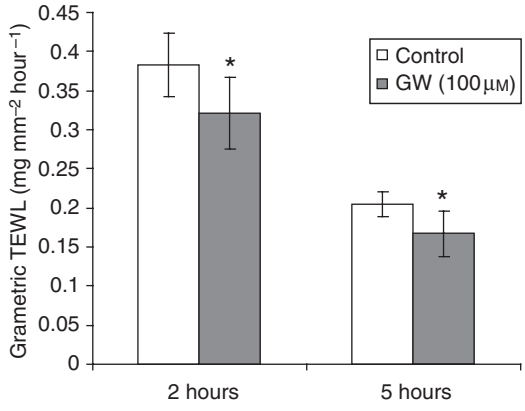

Figure 1. Peroxisome proliferator-activated receptor-δ (PPARδ) activation accelerates epidermal permeability barrier formation. Explants from day 17 fetal rat were incubated in the presence of GW 610742X (100 μM) or vehicle (DMSO) for 2 days. The gravimetric transepidermal water loss (TEWL) (mg mm^{-2} hour^{-1}) of explants was determined as described in the Materials and Methods section. Data are presented as mean ± SD ($n=5$–6; *$P<0.05$), and calculated by subtraction of the weight at 2 hours (TEWL 2 hours) at 5 hours (TEWL 5 hours) from the initial weight at 0 hour. Similar results were obtained when the experiment was repeated twice with epidermal samples prepared from different batches of fetal rats.

To determine whether the accelerated SC formation in fetal explants is accompanied by an increase in the expression of structural proteins (differentiation markers) necessary for corneocyte formation, we next examined the levels of INV and LOR in fetal rat epidermis by immunohistochemistry. Although both INV and LOR were absent in the epidermis from control explants (Supplementary Figure S1, d, g), they were easily detected in explants treated with GW 610742X (Supplementary Figure S1, e-f, h-i). FIL was undetectable in both control and GW 610742X-treated explants (data not shown). Together, our results indicate that activation of PPARδ stimulates the expression of certain structural proteins required for SC formation.

We next evaluated the ultrastructural appearance of the outer epidermis. As shown in Figure 2, although vehicle-treated explants (Figure 2a) again failed to reveal SC formation, SC was apparent in GW 610742X-treated explants (typically two layers) (Figure 2c). Moreover, LB secretion appeared increased in GW 610742X-treated explants (Figure 2d, arrows), and lamellar membranes was evident at the SG–SC interface (Figure 2e, arrows), compared with control (Figure 2b, arrows). These results indicate that PPARδ activation stimulates maturation of both the cellular and extracellular compartments of SC.

To further investigate outside-in barrier function following PPARδ ligand treatment, we next examined the transepidermal diffusion of the fluorescent dye Lucifer yellow in the explants. As shown in Supplementary Figure S2, the dye was found not only to diffuse through all layers of epidermis but was even detectable in the dermis in the control explants (Figure 2a, arrows). In contrast, the dye was mainly restrained in the upper layers of the SC in the GW 610742X-treated explants (Figure 2b, arrows), indicting an accelerated barrier formation in these explants.

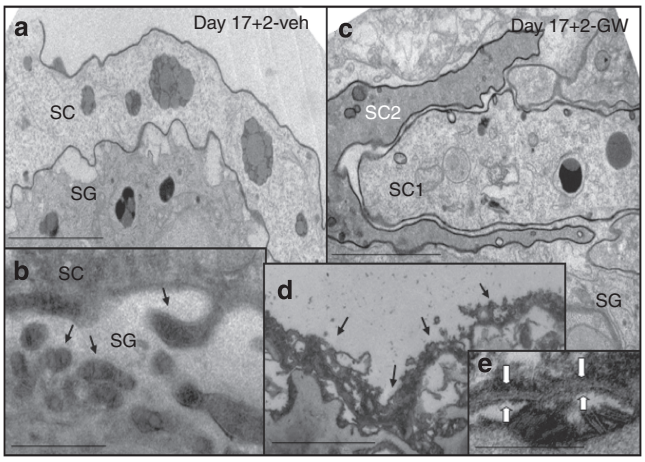

Figure 2. PPARδ activation accelerates stratum corneum formation and epidermal barrier development in fetal rat explants. Explants from day 17 fetal rat were incubated with GW 610742X (100 μM) or vehicle for 2 days, and electron microscope analysis was performed as described in Materials and Methods section. Vehicle control (**a** + **b**). Early stratum corneum (SC) formation is seen, as well as evidence of secretion of lamellar body (LB) contents at the SC–stratum granulosum (SG) interface (arrows). Bar = 1 μm (**a**), 0.2 μm (**b**). GW 610742X-treated (**c-e**). A small increase in SC formation is evident (one more layer of SC; **c**), but a much greater quantity of LB contents are present at SG–SC interface (**d**, arrows). Moreover, there is early evidence of lamellar bilayer formation (**e**, arrows). Note GW 610742X-treated explants display improved LB secretion and post-secretory lipid processing. Bar = 1 μm (**a, c, d**), 0.2 μm (**b**, **e**). Veh: vehicle.

Intra-amniotic administration of PPARδ activator accelerate SC development

We next determined whether the intra-amniotic administration of GW 610742X would accelerate permeability barrier formation *in vivo*. As shown in Figure 3, similar to our results in the fetal skin explant model, intra-amniotic administration of GW 610742X caused a decline in TEWL, indicating that PPARδ activation accelerates permeability barrier formation in the fetal rat skin. We next examined the morphology of the epidermis using light microscopy, and observed that the epidermis of GW 610742X-treated fetuses revealed a more mature epidermis with a multilayered SG and a distinct SC with hematoxylin and eosin (H&E) staining (Supplementary Figure S3, b *vs* a). Similarly, treatment with GW 610742X also increased the expression of INV and LOR in epidermis (Supplementary Figure S3, d, f). FIL was undetectable in both vehicle- and GW 610742X-treated fetal rats (data not shown). In addition, a more mature epidermis, with abundant LB secretion (Figure 4d) and lamellar bilayer formation (Figure 4c) was also evident in the extracellular spaces of the SC of GW 610742X-treated fetal rats by electron microscopy (EM). In contrast, the epidermis from vehicle-treated animals had less LB secretion (Figure 4a), with 1–2 layers of SC (Figure 4b). Finally, when epidermis was stained with nile red, a dye that differentially stains neutral lipids in yellow and polar lipids in red, there were intensive yellow staining in SC layers (Supplementary Figure S4, b) and some red staining in SG layers (Supplementary Figure S4, b, insert) of GW 610742X-treated fetal rat, compared with mostly red staining in vehicle controls (Supplementary Figure S4, a). The hallmark of mature SC is its neutral lipid composition. It is

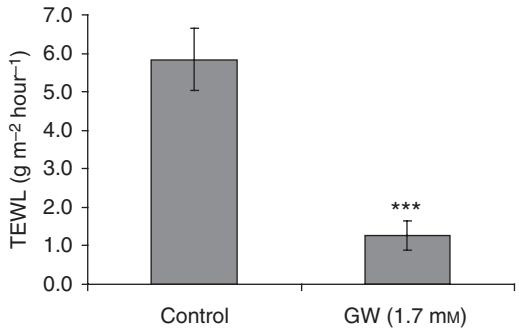

Figure 3. Intra-amniotic administration of PPARδ activator accelerates fetal epidermal permeability barrier formation *in vivo*. At day 17 of gestation, timed-pregnant Sprague–Dawley (SD) rats were injected intra-amniotically with either GW 610742X or vehicle, and transepidermal water loss (TEWL) was measured in fetal rat epidermis on gestational day 19, as described in Materials and Methods section. Results are expressed as the mean ± SD ($n = 7$; ***$P < 0.001$). Similar results were obtained when experiments were repeated with an additional batch of pregnant rats.

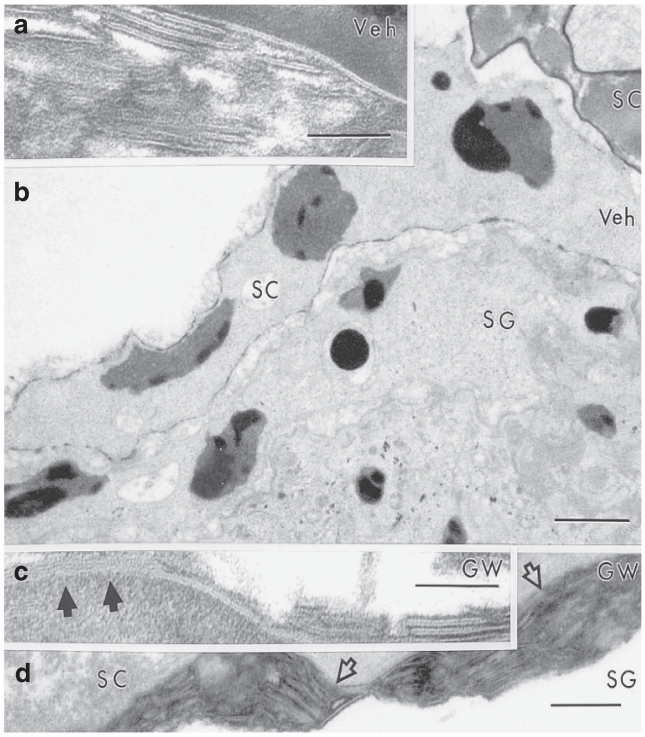

Figure 4. Intra-amniotic administration of PPARδ activator accelerates stratum corneum (SC) formation and epidermal barrier development. Timed-pregnant Sprague–Dawley rats (day 17) were injected intra-amniotically with either GW 610742X or vehicle, and skin samples were collected from fetal rats on gestational day 19 for electron microscopy analysis. Pictures represent electron micrographs of upper epidermis in dorsal skin of fetal rats. Ruthenium tetroxide post-fixation reveals abundant lamellar unit structures in the extracellular spaces of the SC in the epidermis of fetal rats treated with GW 610742X (**d**; open arrows). Early SC formation (**b**) was seen in vehicle control under low magnification; however the lamellar material was shown unorganized (**a**). Mature lamellar bilayers were also presented in GW 610742X-treated rats (**c**; solid arrows). Bar = 0.25 μm (**d**); 0.2μm (**a**, **c**), 1 μm (**b**). Veh: vehicle.

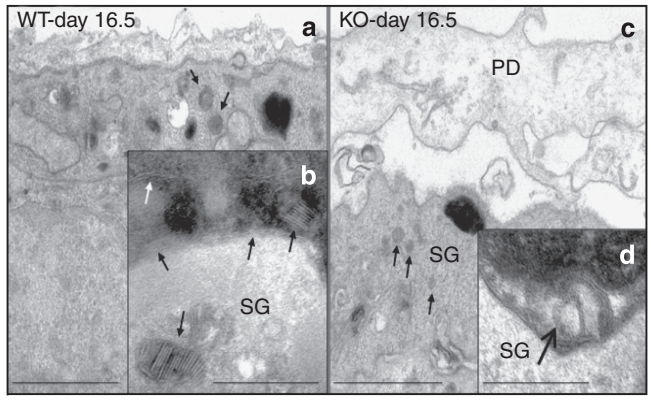

Figure 5. A delay in epidermal development in peroxisome proliferator-activated receptor-δ (PPARδ) knockout (KO) mice at day 16.5 of gestation. Skin biopsies were collected from wild-type (WT) and knockout (KO) fetal mice on day 16.5, and the ultra-structure of epidermis was examined by electron microscopy analysis. KO (**c** + **d**) reduced lamellar bodies (arrows) and secreted contents (arrow) in day 16.5 KO mice. WT (**a** + **b**) stratum corneum (SC)–stratum granulosum (SG) interface of epidermis from day 16.5 of WT mice, showing lamellar bodies (LBs) in SG cytosol (**a**, arrows), and abundant secreted contents above SG (**b**, arrows). Bar = 1 μm (**a-b**); 0.2 μm (**c-d**). PD: periderm.

worth noting that there appeared a slightly increased epidermal thickness on light microscopy of H&E- and nile red-stained sections of fetal rats after amniotic fluid injection with GW 610742X (Supplementary Figure S3, a-b; Supplementary Figure S4). However, this increase is mainly due to the increased layers of SG and SC, because when the distance between the basement lamina and the lower SC was quantified, we did not find any difference in epidermal thickness of the GW 61072X-treated *versus* vehicle control groups (data not shown). Similar phenomena were observed in the epidermis of adult mice topically treated with PPARδ ligand following barrier disruption (Schmuth *et al.*, 2004).

These observations confirm the results in the fetal skin explant model, and together clearly show that PPARδ activation accelerates SC formation and permeability barrier development.

Delayed SC formation in PPARδ-deficient mice

Although the studies described above show that pharmacological activation of PPARδ accelerates fetal epidermal/SC development, they do not address the potential importance of endogenous PPARδ activation for epidermal development. To determine whether PPARδ is an important physiological mediator of SC formation, we next examined epidermal morphology in days 16.5, 17.5, and 18.5 PPARδ knock out (KO) and wild-type (WT) mice by light and EM. At day 16.5 of gestation, PPARδ KO mice displayed a less mature epidermis than WT mice on H&E staining (Supplementary Figure S5, b). Similarly, immunohistochemical staining for LOR also was decreased in the epidermis of PPARδ KO mice (Supplementary Figure S5, f), with slightly decreased INV staining (Supplementary Figure S5, d). It is worth noting that due to its low abundance in epidermis during fetal mouse development, FIL was undetectable under our experimental condition on day 16.5. Finally, EM revealed differences not only in cell layers, but also in the extracellular matrix: while LB secretion was comparable, formation of mature lamellar bilayers was delayed in PPARδ KO (Figure 5c-d) compared with WT (Figure 5a-b) epidermis. Together, these results show a delay in epidermal maturation in day 16.5 PPARδ-deficient mice.

This delay in epidermal maturation persisted in day 17.5 PPARδ KO mice. H&E staining (Supplementary Figure S6, b), immunohistochemical staining for LOR (Supplementary Figure S6, d), and EM (Figure 6), all showed a less mature epidermis in KO compared with WT mice. However, on day 17.5, no difference in the expression levels of INV (data not shown) or FIL (Supplementary Figure S6, e-f) was observed. By day 18.5 and immediately after birth, there were no differences in any parameters (data not shown) between PPARδ KO and WT mice, indicating that the delay in SC formation is transient, occurring primary on days 16.5–17.5, and that with time, SC development normalizes in PPARδ KO mice.

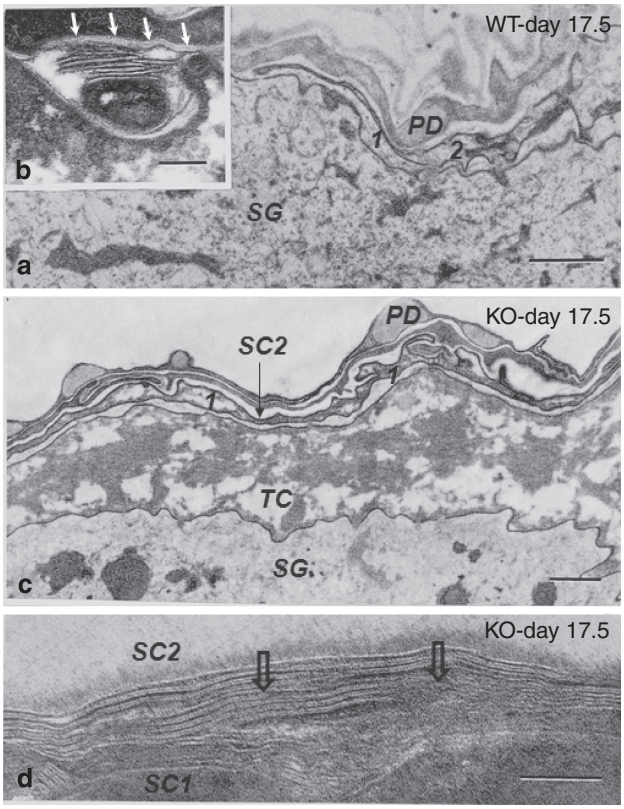

Figure 6. A delay in epidermal development in peroxisome proliferator-activated receptor-δ (PPARδ) knockout (KO) mice persists at day 17.5 of gestation. Skin biopsies were collected from mild-type (WT) and KO mice on day 17.5, and the ultra-structure of epidermis was examined by electron microscope analysis. At 17.5 day, KO epidermis shows delayed maturation of lamellar membranes. (**a**) WT mice show two-layered stratum corneum (SC), and residual periderm (PD) layer. (**b**) (insert): In WT epidermis, secreted lamellar body contents rapidly organized into elongated lamellar bilayers (arrows). (**c** + **d**) In KO epidermis, a two-layered SC and PD are also present, but maturation of secreted lamellar body contents is delayed (only partially-processed lamellae are present) (**d**, open arrows). SG: stratum granulosum, TC: transitional cell. (**a** + **c**) Osmium tetroxide post-fixation; (**b** + **d**) ruthenium-tetroxide fixation. Bar = 1 μm (**a**, **c**); 0.1 μm (**b**, **d**).

DISCUSSION

Peroxisome proliferator-activated receptor-δ is expressed at high levels during embryogenesis in most tissues (Braissant and Wahli, 1998). In the skin, PPARδ is found not only in sebaceous glands, melanocytes, and epidermal Langerhans cells, but also throughout all layers of the epidermis (Braissant and Wahli, 1998). Among the three PPARs, PPARδ is the most abundant isoform in the epidermis (Braissant and Wahli, 1998). During fetal epidermal development, PPARδ is expressed in the epidermis as early as E13.5, and its expression level increases with gestation, reaching a peak at E18.5, which is maintained at a high level until adulthood (Braissant *et al.*, 1996; Braissant and Wahli, 1998; Michalik *et al.*, 2001). The high expression levels of PPARδ during E15.5–18.5, a period of time that coincides with the formation of a competent barrier, and the known importance of PPARδ in epidermal differentiation and permeability barrier homeostasis in adult skin (Westergaard *et al.*, 2001; Schmuth *et al.*, 2004; Kim *et al.*, 2006; Man *et al.*, 2008), suggest a potential role for this receptor in epidermal ontogenesis.

In this study, we show that PPARδ activation both regulates and has a role in epidermal ontogenesis. First, using our well-established fetal rat explant culture model, we showed that treatment with the PPARδ ligand, GW 610742X, accelerates SC formation and permeability barrier development (evidenced by both a decrease in gravimetric TEWL and a blockade of transepidermal diffusion of fluorescence dye Lucifer yellow). The acceleration of permeability barrier development can be attributed to both stimulation of corneocyte formation, and the accelerated appearance of mature lamellar membranes in the SC. Second, the intra-amniotic administration of GW 610742X to pregnant rats also increased epidermal lipid synthesis/deposition, accelerated SC formation and epidermal permeability barrier development, in a manner very similar to what was observed in the cultured fetal explant model. Taken together, these results indicate that activation of PPARδ accelerates epidermal ontogenesis in rodents.

To determine whether PPARδ has an important physiological function in regulating SC formation and permeability barrier development, we studied PPARδ-deficient (KO) fetal mice. We initially compared fetal mice from KO and WT on day 16.5 and observed a delay in the SC formation, cornified envelope structural protein expression (INV and LOR), as well as delayed formation of mature lamellar bilayers in KO fetal epidermis. At gestational day 17.5, this delay in epidermal development persisted, but it was less severe than observed on day 16.5. At a later gestational age (for example, day 18.5) and at birth, there were no apparent differences in SC formation and permeability barrier development in KO *versus* WT mice. These results indicate that although PPARδ has a function in regulating fetal SC formation and permeability barrier development, there are other regulating factors that ultimately compensate for the absence of this nuclear hormone receptor.

The fact that the absence of PPARδ resulted in only a transient delay in SC formation and permeability barrier development was not unexpected. Previous studies have shown that glucocorticoids (Aszterbaum *et al.*, 1992; Hanley *et al.*, 1996a, 1998a), thyroid hormone (Hanley *et al.*, 1996a, 1997a), estrogen (Hanley *et al.*, 1996b), LXR activators (Hanley *et al.*, 1999; Komuves *et al.*, 2002), and PPARα activators (Hanley *et al.*, 1997b, 1999, 2000a), all stimulate SC formation and permeability barrier development. Moreover, in PPARα-deficient mice we also observed only a transient delay in SC formation and permeability barrier development (Schmuth *et al.*, 2002). We would speculate that the presence of multiple pathways that stimulate SC formation and permeability barrier development would allow for the absence of one pathway to be compensated for by other regulators, thus ultimately resulting in a normal SC and permeability barrier, which is required after birth for survival.

There are a number of different mechanisms by which activation of PPARδ could accelerate SC formation and permeability barrier development. First, activation of PPARδ

has been shown to stimulate the expression of proteins, including INV, LOR, FIL, and transglutaminase 1 (Westergaard *et al.*, 2001; Schmuth *et al.*, 2004), required for the differentiation of Kers into corneocytes. The mechanism by which activation of PPARδ stimulates the expression of these differentiation proteins is uncertain, but it could be related to the activation of AP-1 in Kers, as we have shown for LXR (Hanley *et al.*, 2000b). Second, activation of PPARδ stimulates epidermal CHOL, fatty acid, and sphingolipid synthesis (Schmuth *et al.*, 2004; Man *et al.*, 2006), which are required to form LBs (Elias and Menon, 1991; Madison, 2003; Feingold, 2007). Inhibition of either CHOL, fatty acid, or sphingolipid synthesis results in the formation of abnormal LBs and a failure to maintain permeability barrier homeostasis (Feingold *et al.*, 1991; Holleran *et al.*, 1991; Mao-Qiang *et al.*, 1993a, 1993b). Third, activation of PPARδ increases the expression of ABCA12 (Jiang *et al.*, 2008), a protein that is required for the transport of sphingolipids into LBs. The absence of ABCA12 leads to harlequin ichthyosis and lamellar ichthyosis, two devastating skin disorders associated with abnormalities in SC formation and permeability barrier function (Lefevre *et al.*, 2003; Akiyama *et al.*, 2005; Kelsell *et al.*, 2005; Akiyama, 2006). Fourth, activation of PPARδ stimulates LB secretion by SG cells (Man *et al.*, 2006), an essential step in the formation of the extracellular lamellar membranes in the SC required for a normal permeability barrier. Fifth, activation of PPARδ increases β-glucocerebrosidase activity in the SC (Man *et al.*, 2006), which has a key function in converting glucosylceramides to ceramides, which are required for the formation of normal lamellar membranes. A deficiency in β-glucocerebrosidase activity in Gaucher's disease or in KO mice results in the failure to form mature lamellar membranes and abnormal permeability barrier homeostasis (Holleran *et al.*, 1994; Doering *et al.*, 1999; Mizukami *et al.*, 2002). Pertinently, the delayed maturation of lamellar bilayers that was noted in KO mice could reflect decreased lipid processing. Finally, activation of PPARδ upregulates cholesterol sulfotransferase type 2, isoform 1b expression, leading to an increase in CHOL sulfotransferase activity resulting in the enhanced formation of CHOL sulfate (Jiang *et al.*, 2005). Cholesterol sulfate has several key functions in the epidermis, including the stimulation of Ker differentiation and the maintainance of SC cohesion by inhibiting the enzymes that lead to SC desquamation (Epstein *et al.*, 1984; Downing *et al.*, 1993; Denning *et al.*, 1995; Hanley *et al.*, 2001). Thus, activation of PPARδ has numerous potential mechanisms by which it could accelerate SC formation and permeability barrier development (summarized in Figure 7).

In conclusion, this study shows that PPARδ has a function in SC formation and permeability barrier development. Activation of PPARδ accelerates, while the absence of PPARδ delays SC formation and permeability barrier development.

MATERIALS AND METHODS

Materials

Timed-pregnant Sprague–Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA). GW 610742X was a gift from Dr Timothy Willson, Glaxo Smith Kline. The tissue culture medium (M199) was from Cellgro, Mediatech (Herndon, VA), and tissue culture membrane inserts (Transwell-COL, 3 μm pore size) were purchased from Corning Incorporated (Corning, NY). Frozen tissue embedding compound (OCT) was purchased from Miles Scientific Division (Naperville, IL). Affinity-purified rabbit antibodies against INV, LOR, and FIL were purchased from Babco (Berkeley, CA).

Organ culture

Our skin organ culture model has been described previously (Hanley *et al.*, 1997c). Briefly, full-thickness skin explants were removed from estimated gestational age day 17 rat fetuses. Explants were placed dermis side down onto collagen-coated membranes, and submerged in 4 ml of hormone- and serum-free M199 culture media, in the presence or absence of GW 610742X at 50–100 μM (Hanley *et al.*, 1996a).

Intra-amniotic injections

Timed-pregnant Sprague–Dawley rats at gestation day 17 were anesthetized with isoforane and a ventral midline incision was made. The uterine horns were exposed and kept moist with warm saline, and 50 μl of GW 610742X (~1.7 mM) or vehicle (DMSO) was injected into the amniotic fluid space of individual amniotic sacs. Incisions in animals were immediately closed and the pregnancy was allowed to proceed for an additional 48 hours. Fetal rats were collected on day 19 by cesarean section and the dorsal skin were harvested for the analysis.

PPARδ KO mice

The mice used in this study were generated using a similar strategy as described previously (Barak *et al.*, 2002), differing only in that the WT exon 4 was replaced with a β-galactosidase gene, rather than a deletion, upstream of the PPARδ DNA-binding domain (Chawla *et al.*, 2003). Homozygous lacZ knock-in mice (PPARδ $^{ki/ki}$) are backcrossed four generations into the SV129 inbred strain. These mice lack almost the entire PPARδ gene product, including the DNA-binding domain. Animals were maintained on a mouse diet (Ralston-Purina, St Louis, MO) and water *ad lib.* Fetuses were obtained from either homozygous KO or control parent mice, and the day of vaginal plug was considered as day 0.5. Pups were delivered by cesarean sections on days 16.5, 17.5, and 18.5 of gestation, and their weights were carefully recorded and matched between WT and KO fetal mice (difference is <0.3 g for morphology study and <0.1 g for EM study). Data presented are from three independent experiments (three breedings); each breeding includes two KO mice and two WT mice for each time point (days 17, 18, and new born).

Immunohistochemistry

Tissue samples were fixed in 10% formaldehyde (freshly prepared from paraformaldehyde) in phosphate-buffered saline for 24 hours and were embedded in paraffin. Sections (5 μm) were collected on positively charged microscope slides (SuperfrostPlus, Fisher Scientific, Pittsburgh, PA) and incubated overnight with antibodies, specific for INV (1:1000), LOR (1:1500), or FIL (1:3000) (Komuves *et al.*, 1999).

Microscopy and imaging

Frozen or paraffin-embedded skin sections (5 μm) were stained with H&E or nile red (Aszterbaum *et al.*, 1992), or used for immunohis-

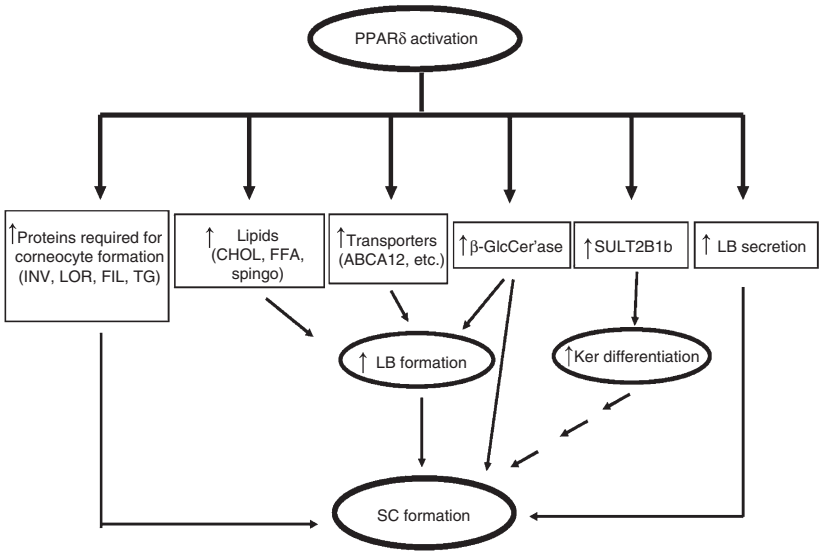

Figure 7. Potential mechanisms by which peroxisome proliferator-activated receptor-δ (PPARδ) activation accelerates stratum corneum (SC) formation and epidermal permeability barrier ontogenesis.

tochemistry. Subsequently, slides were examined with a Zeiss light microscope (Axioplan 2, Munich, Germany) (for H&E or immunohistochemistry), or a Leitz fluorescence microscope equipped for epifluorescence (for nile red), and digital images were captured using AxioVision software (Zeiss Vision, München, Hallbergmoons, Germany).

Electron microscopy

Parallel samples were minced into 1-mm^3 pieces, fixed in modified Karnovsky's fixative, and processed for EM as described previously (Hou *et al*, 1991). Ultrathin sections (60 nm) were contrasted with uranyl acetate and lead citrate, and examined with a Zeiss 10 Åelectron microscope (Carl Zeiss, Thornwood, NY) operated at 60 kV.

Permeability barrier function

In vitro gravimetric TEWL. At the end of incubation, each skin explant was removed and cut into a single piece using an Acu-Punch (5–6-mm in diameter) (Acuderm, Ft Lauderdale, FL) to ensure an equal area for analysis. The sides and bottoms of the tissues were carefully sealed to restrict water loss to solely through the epidermis, as described previously (Nolte *et al*., 1993). Samples were then weighted at 2 and 5 hours using a Cahn balance (sensitivity 0.0001 mg).

Transepidermal outside-in barrier function. Following the treatment, explants were physically fixed in Petri dishes with edges sealed, and incubated with 1 mM Lucifer yellow dye in phosphate-buffered saline (pH 7.4) at 37 °C for 1 hour. Explants were then rinsed, frozen and sectioned. The sections (5 μm) were analyzed by fluorescence microscopy.

In vivo TEWL. Following intra-amniotic injections, fetal rats were collected on day 19 by cesarean section and TEWL were measured with an electrolytic water analyzer (TEWAMETER 300, Köln, Germany).

Statistical analysis

All data are expressed as mean ± SEM. Comparison between two groups is undertaken using two-tail and unpaired *t*-test. Differences in values are considered significant if $P<0.05$.

INSTITUTIONAL APPROVAL

The use of animals and all experimental procedures were approved by the appropriate committees including the Institutional Animal Care and Use Committee of the San Francisco Veterans Affairs Medical Center.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Akiyama M (2006) Pathomechanisms of harlequin ichthyosis and ABCA transporters in human diseases. *Arch Dermatol* 142:914–8

Akiyama M, Sugiyama-Nakagiri Y, Sakai K, McMillan JR, Goto M, Arita K *et al.* (2005) Mutations in lipid transporter ABCA12 in harlequin ichthyosis and functional recovery by corrective gene transfer. *J Clin Invest* 115:1777–84

Aszterbaum M, Feingold KR, Menon GK, Williams ML (1993) Glucocorticoids accelerate fetal maturation of the epidermal permeability barrier in the rat. *J Clin Invest* 91:2703–8

Aszterbaum M, Menon GK, Feingold KR, Williams ML (1992) Ontogeny of the epidermal barrier to water loss in the rat: correlation of function with stratum corneum structure and lipid content. *Pediatr Res* 31:308–17

Barak Y, Liao D, He W, Ong ES, Nelson MC, Olefsky JM *et al.* (2002) Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. *Proc Natl Acad Sci USA* 99:303–8

Bickenbach JR, Greer JM, Bundman DS, Rothnagel JA, Roop DR (1995) Loricrin expression is coordinated with other epidermal proteins and the appearance of lipid lamellar granules in development. *J Invest Dermatol* 104:405–10

Braissant O, Foufelle F, Scotto C, Dauca M, Wahli W (1996) Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* 137:354–66

Braissant O, Wahli W (1998) Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. *Endocrinology* 139:2748–54

Cartlidge PH, Stewart JH (1995) Effect of changing the stillbirth definition on evaluation of perinatal mortality rates. *Lancet* 346:486–8

Chawla A, Lee CH, Barak Y, He W, Rosenfeld J, Liao D *et al.* (2003) PPARdelta is a very low-density lipoprotein sensor in macrophages. *Proc Natl Acad Sci USA* 100:1268–73

Denning MF, Kazanietz MG, Blumberg PM, Yuspa SH (1995) Cholesterol sulfate activates multiple protein kinase C isoenzymes and induces granular cell differentiation in cultured murine keratinocytes. *Cell Growth Differ* 6:1619–26

Doering T, Proia RL, Sandhoff K (1999) Accumulation of protein-bound epidermal glucosylceramides in beta-glucocerebrosidase deficient type 2 Gaucher mice. *FEBS Lett* 447:167–70

Downing DT, Dose RW, Abraham W (1993) Interaction between sphingosine and cholesteryl sulfate in epidermal lipids. *J Lipid Res* 34:563–9

Eckert RL, Crish JF, Robinson NA (1997) The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol Rev* 77:397–424

Elias PM, Menon GK (1991) Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv Lipid Res* 24:1–26

Epstein EH, Williams ML, Elias PM (1984) The epidermal cholesterol sulfate cycle. *J Am Acad Dermatol* 10:866–8

Feingold KR (2007) Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. *J Lipid Res* 48:2531–46

Feingold KR, Man MQ, Proksch E, Menon GK, Brown BE, Elias PM (1991) The lovastatin-treated rodent: a new model of barrier disruption and epidermal hyperplasia. *J Invest Dermatol* 96:201–9

Foster CA, Bertram JF, Holbrook KA (1988) Morphometric and statistical analyses describing the *in utero* growth of human epidermis. *Anat Rec* 222:201–6

Fuchs E (1990) Epidermal differentiation: the bare essentials. *J Cell Biol* 111:2807–14

Hammarlund K, Sedin G (1979) Transepidermal water loss in newborn infants. III. Relation to gestational age. *Acta paediatrica Scandinavica* 68:795–801

Hanley K, Devaskar UP, Hicks SJ, Jiang Y, Crumrine D, Elias PM *et al.* (1997a) Hypothyroidism delays fetal stratum corneum development in mice. *Pediatr Res* 42:610–4

Hanley K, Feingold KR, Komuves LG, Elias PM, Muglia LJ, Majzoub JA *et al.* (1998a) Glucocorticoid deficiency delays stratum corneum maturation in the fetal mouse. *J Invest Dermatol* 111:440–4

Hanley K, Jiang Y, Crumrine D, Bass NM, Appel R, Elias PM *et al.* (1997b) Activators of the nuclear hormone receptors PPARalpha and FXR accelerate the development of the fetal epidermal permeability barrier. *J Clin Invest* 100:705–12

Hanley K, Jiang Y, Elias PM, Feingold KR, Williams ML (1997c) Acceleration of barrier ontogenesis *in vitro* through air exposure. *Pediatr Res* 41:293–9

Hanley K, Jiang Y, He SS, Friedman M, Elias PM, Bikle DD *et al.* (1998b) Keratinocyte differentiation is stimulated by activators of the nuclear hormone receptor PPARalpha. *J Invest Dermatol* 110:368–75

Hanley K, Komuves LG, Bass NM, He SS, Jiang Y, Crumrine D *et al.* (1999) Fetal epidermal differentiation and barrier development *In vivo* is accelerated by nuclear hormone receptor activators. *J Invest Dermatol* 113:788–95

Hanley K, Komuves LG, Ng DC, Schoonjans K, He SS, Lau P *et al.* (2000a) Farnesol stimulates differentiation in epidermal keratinocytes via PPARalpha. *J Biol Chem* 275:11484–91

Hanley K, Ng DC, He SS, Lau P, Min K, Elias PM *et al.* (2000b) Oxysterols induce differentiation in human keratinocytes and increase Ap-1-dependent involucrin transcription. *J Invest Dermatol* 114:545–53

Hanley K, Rassner U, Elias PM, Williams ML, Feingold KR (1996a) Epidermal barrier ontogenesis: maturation in serum-free media and acceleration by glucocorticoids and thyroid hormone but not selected growth factors. *J Invest Dermatol* 106:404–11

Hanley K, Rassner U, Jiang Y, Vansomphone D, Crumrine D, Komuves L *et al.* (1996b) Hormonal basis for the gender difference in epidermal barrier formation in the fetal rat. Acceleration by estrogen and delay by testosterone. *J Clin Invest* 97:2576–84

Hanley K, Rassner U, Jiang Y, Vansomphone D, Crumrine D, Komuves L *et al.* (1996c) Hormonal basis for the gender difference in epidermal barrier formation in the fetal rat. Acceleration by estrogen and delay by testosterone. *J Clin Invest* 97:2576–84

Hanley K, Wood L, Ng DC, He SS, Lau P, Moser A *et al.* (2001) Cholesterol sulfate stimulates involucrin transcription in keratinocytes by increasing Fra-1, Fra-2, and Jun D. *J Lipid Res* 42:390–8

Hardman MJ, Sisi P, Banbury DN, Byrne C (1998) Patterned acquisition of skin barrier function during development. *Development* 125:1541–52

Harpin VA, Rutter N (1983) Barrier properties of the newborn infant's skin. *J Pediatr* 102:419–25

Holleran WM, Ginns EI, Menon GK, Grundmann JU, Fartasch M, McKinney CE *et al.* (1994) Consequences of beta-glucocerebrosidase deficiency in epidermis. Ultrastructure and permeability barrier alterations in Gaucher disease. *J Clin Invest* 93:1756–64

Holleran WM, Man MQ, Gao WN, Menon GK, Elias PM, Feingold KR (1991) Sphingolipids are required for mammalian epidermal barrier function. Inhibition of sphingolipid synthesis delays barrier recovery after acute perturbation. *J Clin Invest* 88:1338–45

Hou SY, Mitra AK, White SH, Menon GK, Ghadially R, Elias PM (1991) Membrane structures in normal and essential fatty acid-deficient stratum corneum: characterization by ruthenium tetroxide staining and x-ray diffraction. *J Invest Dermatol* 96:215–23

Jiang YJ, Kim P, Elias PM, Feingold KR (2005) LXR and PPAR activators stimulate cholesterol sulfotransferase type 2 isoform 1b in human keratinocytes. *J Lipid Res* 46:2657–66

Jiang YJ, Lu B, Kim P, Paragh G, Schmitz G, Elias PM *et al.* (2008) PPAR and LXR activators regulate ABCA12 expression in human keratinocytes. *J Invest Dermatol* 128:104–9

Kelsell DP, Norgett EE, Unsworth H, Teh MT, Cullup T, Mein CA *et al.* (2005) Mutations in ABCA12 underlie the severe congenital skin disease harlequin ichthyosis. *Am J Hum Genet* 76:794–803

Kim DJ, Bility MT, Billin AN, Willson TM, Gonzalez FJ, Peters JM (2006) PPARbeta/delta selectively induces differentiation and inhibits cell proliferation. *Cell Death Differ* 13:53–60

Komuves LG, Hanley K, Jiang Y, Katagiri C, Elias PM, Williams ML *et al.* (1999) Induction of selected lipid metabolic enzymes and differentiation-linked structural proteins by air exposure in fetal rat skin explants. *J Invest Dermatol* 112:303–9

Komuves LG, Hanley K, Lefebvre AM, Man MQ, Ng DC, Bikle DD *et al.* (2000) Stimulation of PPARalpha promotes epidermal keratinocyte differentiation *in vivo*. *J Invest Dermatol* 115:353–60

Komuves LG, Schmuth M, Fowler AJ, Elias PM, Hanley K, Man MQ *et al.* (2002) Oxysterol stimulation of epidermal differentiation is mediated by liver X receptor-beta in murine epidermis. *J Invest Dermatol* 118:25–34

Kuenzli S, Saurat JH (2003) Peroxisome proliferator-activated receptors in cutaneous biology. *Br J Dermatol* 149:229–36

Lefevre C, Audebert S, Jobard F, Bouadjar B, Lakhdar H, Boughdene-Stambouli O *et al.* (2003) Mutations in the transporter ABCA12

are associated with lamellar ichthyosis type 2. *Hum Mol Genet* 12:2369–78

Madison KC (2003) Barrier function of the skin: ''la raison d'etre'' of the epidermis. *J Invest Dermatol* 121:231–41

Man MQ, Barish GD, Schmuth M, Crumrine D, Barak Y, Chang S *et al.* (2008) Deficiency of PPARbeta/delta in the epidermis results in defective cutaneous permeability barrier homeostasis and increased inflammation. *J Invest Dermatol* 128:370–7

Man MQ, Choi EH, Schmuth M, Crumrine D, Uchida Y, Elias PM *et al.* (2006) Basis for improved permeability barrier homeostasis induced by PPAR and LXR activators: liposensors stimulate lipid synthesis, lamellar body secretion, and post-secretory lipid processing. *J Invest Dermatol* 126:386–92

Mao-Qiang M, Elias PM, Feingold KR (1993a) Fatty acids are required for epidermal permeability barrier function. *J Clin Invest* 92:791–8

Mao-Qiang M, Feingold KR, Elias PM (1993b) Inhibition of cholesterol and sphingolipid synthesis causes paradoxical effects on permeability Barrier Homeostasis. *J Invest Dermatol* 101:185–90

Michalik L, Desvergne B, Tan NS, Basu-Modak S, Escher P, Rieusset J *et al.* (2001) Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)alpha and PPARbeta mutant mice. *J Cell Biol* 154:799–814

Mizukami H, Mi Y, Wada R, Kono M, Yamashita T, Liu Y *et al.* (2002) Systemic inflammation in glucocerebrosidase-deficient mice with minimal glucosylceramide storage. *J Clin Invest* 109: 1215–21

Nolte CJ, Oleson MA, Bilbo PR, Parenteau NL (1993) Development of a stratum corneum and barrier function in an organotypic skin culture. *Arch Dermatol Res* 285:466–74

Schmuth M, Haqq CM, Cairns WJ, Holder JC, Dorsam S, Chang S *et al.* (2004) Peroxisome proliferator-activated receptor (PPAR)-beta/delta stimulates differentiation and lipid accumulation in keratinocytes. *J Invest Dermatol* 122:971–83

Schmuth M, Jiang YJ, Dubrac S, Elias PM, Feingold KR (2008) Thematic review series: skin lipids. Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology. *J Lipid Res* 49:499–509

Schmuth M, Schoonjans K, Yu QC, Fluhr JW, Crumrine D, Hachem JP *et al.* (2002) Role of peroxisome proliferator-activated receptor alpha in epidermal development *in utero*. *J Invest Dermatol* 119:1298–303

Westergaard M, Henningsen J, Svendsen ML, Johansen C, Jensen UB, Schroder HD *et al.* (2001) Modulation of keratinocyte gene expression and differentiation by PPAR-selective ligands and tetradecylthioacetic acid. *J Invest Dermatol* 116:702–12